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(54) Title: CONTROL OF ANGIOGENESIS AND COMPOSITIONS AND METHODS THEREFOR

(57) Abstract

Synthetic peptides which are active in stimulating angiogenesis in animals are disclosed. The peptides have sequences substantially corresponding to amino acid sequences that occur in epidermal growth factor. Compositions including these synthetic peptides and a method for the stimulation of angiogenesis in animals are also disclosed.

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"CONTROL OF ANGIOGENESIS AND COMPOSITIONS AND METHODS THEREFOR"

FIELD OF THE INVENTION

This invention relates to the control of angiogenesis, and to methods and compositions therefor using synthetic peptides having amino acid sequences corresponding to sequences found in epidermal growth factor.

BACKGROUND TO THE INVENTION

Angiogenesis is the development of blood vessels. The identification of agents which will permit control of angiogenesis is of considerable interest in medical science and to associated industries; substances which stimulate angiogenesis may, for instance, have value in promoting wound healing, while inhibitors of angiogenesis could find application for retarding tumor growth or blocking the onset of diabetic blindness.

Angiogenic properties are exhibited by a variety of agents. Thus, the induction of new blood vessel growth or formation of a vascular network is elicited in animals by agents such as extracts of carcinoma cells or of normal bovine parotid glands; partially purified fractions of substances from Walker carcinoma cells, bovine parotid cells or bovine liver have been shown to be angiogenic by occular implant and chick chorioallantoic membrane assays. It has also been shown that low concentrations of copper ions can induce neovascularisation in the anterior eye chamber or corneal pocket.

Unfortunately, none of the aforementioned substances are currently acceptable clinically and it is not clear whether they act directly on the vascular system.

While there is, therefore, a need for angiogenic agents which are more medically and/or economically acceptable than those considered hereto, the identification of such agents has been hindered by an

imperfect understanding of the mechanisms of angiogenesis.

Following reports that epidermal growth factor (EGF) provokes angiogenesis, in vitro and in vivo experiments were conducted which confirm this. EGF itself is not, however, an attractive candidate as an angiogenic agent since from natural sources, it is not available in quantities which would meet widespread demand, and being a peptide chain 53 amino acids in length, it has not yet been found possible to synthesize EGF economically on a commercial scale.

DESCRIPTION OF THE INVENTION

It has been established by the inventor that angiogenic activity is exhibited by certain synthetic peptides corresponding in basic structure to fragments (amino acid-sequences) of EGF, which by virtue of their relative shortness present substantially fewer problems of synthesis than the entire EGF molecule.

Accordingly, in one aspect of this invention there is provided a method for stimulating angiogenesis in animals characterised by the step of administering to an animal an effective amount of an angiogenically stimulating synthetic peptide having an amino acid sequence substantially corresponding to an amino acid sequence occurring in EGF, excluding EGF. The term amino acid is to be understood to embrace amino acid substitutes as recognized in the art.

In another aspect, the present invention provides a synthetic peptide, which is active in stimulating angiogenesis in animals, having an amino acid sequence substantially corresponding to an amino acid sequence occurring in epidermal growth factor (EGF), excluding EGF.

In a still further aspect, the present invention provides a composition for use in the stimulation of angiogenesis comprising an effective amount of a synthetic peptide, which is active in stimulating angiongenesis,

having an amino acid sequence substantially corresponding to an amino acid sequence occurring in epidermal growth factor (EGF), excluding EGF, and a pharmaceutically acceptable carrier.

The active sequences may be identified using a cell migration assay as a screen system, together with an in vivo assay that minimises inflammation. The present inventor has using these techniques, identified synthetic peptide known amino acid sequences that induce angiogenesis by a medium involving (or consistent with) direct action on endothelial cells. The amino acid sequence for naturally occurring EGF is shown in Figure 1.

Those sequences identified as active are:

3 - 14

3 - 10

12 - 14

12 - 15

29 - 37

33 - 37

Analogues or derivatives of these sequences, including small sub-fragments are also included in the scope of the invention.

MODES FOR CARRYING OUT THE INVENTION

In order that the invention may be more readily understood reference will now be made to experimental procedures and results.

Identification of Active Sequences

Materials and Methods

Polymer Preparation

A slow release co-polymer of ethylene-vinyl acetate (Elvax 60; trademark of Polysciences Corp.) was prepared by the method of Langer and Folkman 1977. (Nature 263 797-800).

Endothelial Cells

A line of capillary endothelial cells free from neural cells was prepared from bovine retinas McAuslan et al 1986

(In Vitro Models for Cancer Research. Chemical Rubber Co. Publication).

Cell Migration Assay

The procedures for studying induced endothelial cell migration as well as the quantitation of track area or length were as presented in detail by McAuslan and Reilley 1980 (Exptl Cell Res. 130 147-157).

All peptides were dissolved in serumless medium 199 at 37°C for 3 hours, and were all used at a concentration of 100ng/ml. The experimental medium consisted of M199 and 5% Foetal Calf Serum. Measurements of area and length were made using a Bioquant Image Analysis system, and mean values were computed for 150 cell tracks taken from triplicate dishes.

All cell lines for experimentation were used between the 3rd and 4th passage.

Epidermal Growth Factor was used in the form described by McAuslan 1985 (Cell Biol. Int. Reports 9 175-182). The molecular species were further purified by reverse phase high performance liquid chromatography. EGF fragments were synthesised using a combined chemical-enzyme procedure described by Widmer et al in "Peptides 1984" pp14 Almquist and Wiksell International, Stockholm.

EGF fragments found to be active had the following structures:

Ac (3-10) NH_2 i.e.

Ac. Tyr. Pro. Gly. Cys (ACM). Pro. Ser. Ser. Tyr. NH_2.

Ac (12-14) NH_2 i.e.

H. Gly. Tyr. Cys. (ACM) NH_2.

BOC (12-15) OH i.e.

BOC. Gly. Tyr. Cys. (ACM) Leu. OH.

AC (3-14) NH_2 i.e.

Ac. Tyr. Pro. Gly. Cys (ACM). Pro. Ser. Ser. Tyr. Asp. Gly. Tyr. (ACM). NH_2.

(29-37) i.e.

Ac. Tyr. Thr. Gly. Asn. Cys. (ACM). Val. Ile. Gly. Tyr. Oet.

(33-37) i.e.

Bz. Arg. Cys (ACM). Val. Ile. Gly. Tyr. OMe.

Where:

Oet = oxyethyl ester.

Ac = acetyl.

ACM = aceto amido methyl.

H = free amino group.

BOC = tertiary butyl oxycarbonyl.

NH_2 = amide on a carboxyl terminal group.

OMe = oxymethyl ester.

OH = free carboxyl terminal.

BZ = benzoyl.

and conventional abbreviations are used for amino acid moieties.

DETERMINATION OF ANGIOGENIC ACTIVITY

Sub-Cutaneous Implant Assay for Angiogenic Activity

Fragments ($1mm^3$) of Elvax polymer were impregnated with saturating amounts (0.5mg) of the solid form of test substances. The so-formed pellets were sterilised by ultra-violet irradiation and then imbedded in a gel of non-inflammatory atelo-collagen type I (final pH 6.5, 40mg collagen/ml saline). This in turn was contained in a shallow cylinder of silastic tubing (4 x 3 x 2mm), to provide a slow diffusion of test material to surrounding tissue when the device was implanted sub-cutaneously between dorsal dermal layer and muscle fascia of test NZ white rabbits, in such a way as to cause no trauma to the vascular system. Controls showed no visible inflammatory response and no signs of inducing neovasculogenesis in surrounding tissue.

After 10 days implants were examined in situ, then excised and examined microscopically and histologically.

In the case of certain test materials there was obvious development of a microvascular system which grew from the surrounding tissue towards and often into the implanted device. There was no overt inflammation produced by the test materials.

For each tested substance there were two implants per rabbit, one, on the left and one on the right dorsal side.

Results

The results are presented at Tables 1 and 2.

From these results it is evident that the sequences identified have substantial angiogenic activity.

The angiogenic compounds according to this invention may find application in a variety of clinical fields. They may be used, for instance to enhance the healing of burns and wounds (especially those involving tissue

defects); (ii) to promote the acceptance of skin or organ grafts; (iii) in reconstructive and cosmetic surgery (including subdermal implants); and (iv) in prosthetic surgery, particularly where involved in vascular prosthesis.

The compounds may be administered singly, in association with each or in association with angiogenic stimulator in different molecular species. Additionally, they are suitable for release from biodegradable matrices and by slow-release techniques.

TABLE 1

Migration Assay

Peptide	Mean Area ($x10^{-3}$ m^2)	Mean Length (m)	% Increase
Ac (3-10)NH_2	54.3	327	87
BOC (12-15) OH	54.3	329	87
Ac (3-14) NH_2	48.5	304	67
Ac (12-14)NH_2	35.0	302	50
(29-37)	48.5	307	68
(33-37)	52.6	319	76
EGF_C	86.2	508	200
CONTROL	28.5	213	0

TABLE 2

In Vivo Angiogenesis

Peptide	Number of Implants/Intensity of Vascularisation.				
	++++	+++	++	+	-
CONTROL	0	0	1	1	10
EGF_C	5	4	3	0	4
Ac (3-14) NH_2	4	5	1	1	1
Ac (3-10) NH_2	4	2	0	0	0
Act (12-14) NH_2	2	1	4	0	5
BOC (12-15) OH	0	2	2	0	2
(29-37)	0	2	1	1	2
(33-37)	0	2	2	1	1

Intensity of Vascularisation:

++++ (1) Large numbers of distinct capillary vessels invading the gel; numerous blood vessels growing towards the tube. Markedly angiogenic.

+++ (1) Fine blood vessels invading the gel. Less intense than above. Fine blood vessels around the silicon tube. Strongly angiogenic.

++ (1) Slight pink around the periphery of the gel due to few fine capillaries; fine blood vessels around the silicon tube. Weakly angiogenic.

\+ (1) Collagen gel unchanged.

(2) Fine blood vessels growing toward the silicon tube. Incipient angiogenesis.

\- (1) Collagen gel unchanged. No blood vessels around the silicon tube. Non-angiogenic.

CLAIMS

1. A synthetic peptide, which is active in stimulating angiogenesis in animals, having an amino acid sequence substantially corresponding to an amino acid sequence occurring in epidermal growth factor (EGF), excluding EGF.

2. A peptide as in claim 1 and having the sequence Ac. Tyr. Pro. Gly. Cys (ACM). Pro. Ser. Ser. Tyr. NH_2.

3. A peptide as in claim 1 and having the sequence H. Gly. Tyr. Cys. (ACM) NH_2.

4. A peptide as in claim 1 and having the sequence BOC. Gly. Tyr. Cys. (ACM) Leu. OH.

5. A peptide as in claim 1 and having the sequence Ac. Tyr. Pro. Gly. Cys (ACM). Pro. Ser. Ser. Tyr. Asp. Gly. Tyr. (ACM). NH_2.

6. A peptide as in claim 1 and having the sequence Ac. Tyr. Thr. Gly. Asn. Cys. (ACM). Val. Ile. Gly. Tyr. Oet.

7. A peptide as in claim 1 and having the sequence Bz. Arg. Cys (ACM). Val. Ile. Gly. Tyr. OMe.

8. A composition for use in the stimulation of angiogenesis comprising an effective amount of a synthetic peptide as claimed in any one of claims 1 to 7 and a pharmaceutically acceptable carrier.

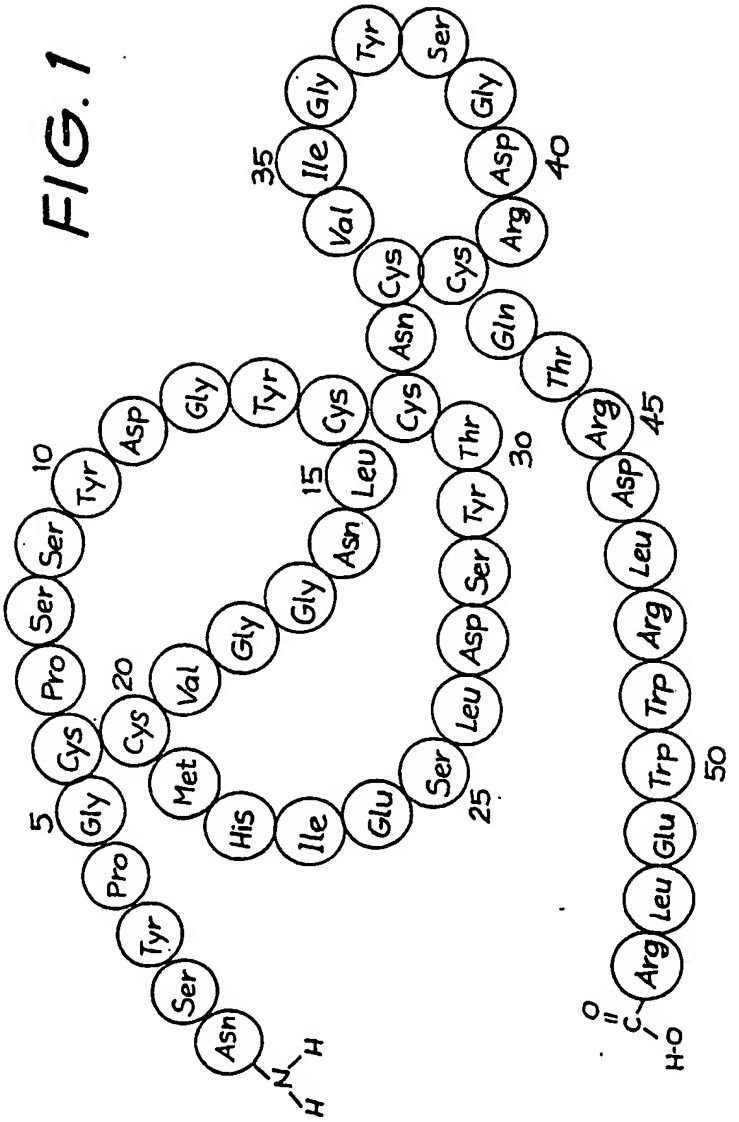
9. A composition as in claim 8 including an angiogenic stimulating compound of a different molecular species.

10. A composition as in claim 8 or claim 9 in the form of a slow release composition.

11. A method for stimulating angiogenesis in animals characterized by the step of administering to an animal an effective amount of synthetic peptide as claimed in any one of claims 1 to 7.

12. A method for stimulating angiogenesis in animals characterized by the step of administering to an animal an effective amount of a composition as claimed in claim 8 or claim 9 or claim 10.

FIG. 1


Asn Ser Tyr Pro Gly Cys Cys Val Gly Met His Ile Glu Ser Leu Asp Ser Tyr Thr Cys Cys Asn Leu Gly Gly Asn Tyr Asp Gly Ser Ser Tyr Cys Cys Val Ile Gly Tyr Ser Gly Asp Arg Cys Cys Gln Thr Arg Asp Leu Arg Trp Trp Glu Leu Arg
5 10 15 20 25 30 35 40 45 50
H-N-H
O=C-OH

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 88/00300

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07K 5/08, 5/10, 7/06, 7/10, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	WPI,WPIL, USPA : Keywords : "EPIDERMAL GROWTH FACTOR" (Derwent Database)

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

AU: IPC as above. GenBank, Kyoto, EMBL, NBRF Databases
Chemical Abstracts: 1983-1988: Molecular and Molecular Fragment Formulas

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	US,A, 4743679 (COHEN, C.M. and R. CREA) 10 May 1988 (10.05.88)	(1)
P,X	Derwent WPI/L Online Abstract Accession no. 88-136324/20, JP,A, 63-077823 (FUJISAWA PHARM K.K.) 8 April 1988 (08.04.88)	(1)
P,X	Derwent WPI/L Online Abstract Accession no. 88-046257/07, JP,A, 63-003791 (HITACHI K.K. and HITACHI CHEMICAL K.K.) 8 January 1988 (08.01.88)	(1)
P,X	US,A, 4686283 (NESTOR, Jr., J.J. and A.B. SCHREIBER) 11 August 1987 (11.08.87)	(1)
X	Patent Abstracts of Japan, C-470, page 12, JP,A, 62-17402 (WAKUNAGA PHARMACEUT CO LTD; KAKIMOTO, M.) 30 July 1987 (30.07.87)	(1,8,10)
X	Patent Abstracts of Japan, C-463, page 118, JP,A, 62-149622 (WAKUNAGA PHARMACEUT CO LTD; UDA, N.) 3 July 1987 (03.07.87)	(1,8,10)
X	EP,A, 224885 (WAKUNAGA SEIYAKU KABUSHIKI KAISHA; AMAGASE, H. et al) 10 June 1987 (10.06.87)	(1,8)

(continued)

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 November 1988 (16.11.88)	24 NOVEMBER 1988 (24.11.88)
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	J.H. CHAN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	EP,A, 205051 (ZAMBON S.P.A.; GAZZANIGA, A. et al) 17 December 1986 (17.12.86)	(1,8)
X	AU,A, 34390/84 (KOMORIYA, A. et al) 28 March 1985 (28.03.85)	(1,8,10)
X	WO,A, 8500369 (CHIRON CORPORATION; BELL, G.) 31 January 1985 (31.01.85)	(1)
X	US,A, 4490365 (PANARETTO, B.A. et al) 25 December 1984 (25.12.84)	(1)
X	Patent Abstracts of Japan, C-225, page 70, JP,A, 59-27858 (NIPPON SHINYAKU K.K.; YAJIMA, H.) 14 February 1984 (14.02.84) (continued)	(1)

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 11&12 because they relate to subject matter not required to be searched by this Authority, namely:

they are excluded subject matter under Rule 39, that is methods of treatment of the animal body.

2. [X] Claim numbers 1, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the claim as drafted is speculative with regard to the term synthetic peptide. Therefore the search was performed on the compounds defined by claims 2 to 7 with regards to Chemical Abstracts.

3. ☐ Claim numbers ____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	US,A, 3948875 (COHEN, S. et al) 6 April 1976 (06.04.76)	(1)
X	US,A, 3917824 (CAMBLE, R. et al) 4 November 1975 (04.11.75)	(1,8)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 88/00300

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
US 4743679	AU 69011/87	EP 234888	JP 62246600
EP 224885	JP 63107941	JP 63099017	
EP 205051	JP 62000027		
AU 34390/84	WO 8501284	EP 156899	IL 69719
WO 8500369	EP 148922		
US 4490365	ES 504434	ES 8304922	ES 519233
	FR 2487677	GB 2082186	NZ 197903
	TR 21384	ZA 8105349	AU 73659/81
US 3917824	AU 65949/74	BE 812973	CA 1026673
	DE 2415101	FR 2223033	GB 1417776
	IL 44288	ZA 7401050	

END OF ANNEX